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CONFERENCE ON PROBLEMS OF GENERAL AND CELLULAR PHYSIOLOGY RELATING TO FERTILIZATION. III.[1]

ION EXCHANGES AND FERTILIZATION IN ECHINODERM EGGS[2]

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THIS lecture summarizes work initiated by the second author and continued over several years by the two authors particularly on *Arbacia punctulata* (Chambers and Chambers, 1938, 1939; Chambers, 1946). Further investigation on the subject was done by the first author in conjunction with the late Dr. S. C. Brooks, using the eggs of two species of *Strongylocentrotus* with radioactive ions as tracers (Chambers *et al.*, 1948; Chambers, 1949).

The experiments constitute an analysis of the changes in susceptibility of the developing sea urchin egg to isotonic potassium chloride. These data are considered in the light of studies on the rate of exchange of potassium and sodium in the sea urchin egg.

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[2] Aided by a grant from the National Cancer Institute, U. S. Public Health Service.

1. Effect of KCl on the Unfertilized and Fertilized Eggs of *Arbacia punctulata*

Eggs were transferred to isotonic KCl, unbuffered, at pH 6.0, 18° C., through several washes by centrifugation. Unfertilized eggs were immersed in the KCl, and returned to sea water at intervals of time and tested for their viability by inseminating them and securing the percentage cleavages. After 8 hours' immersion in KCl, 98 per cent. of the unfertilized eggs were fertilizable and then cleaved. Longer sojourns resulted in increasingly lower percentages of cleavage.

Transference of the fertilized eggs to KCl caused at certain times a complete cessation of further development and not at others. In accordance with the reaction observed on immersion three definite periods were found—an early and a late susceptible period characterized by inhibition of development, and an intermediate resistant period during which development continued. A remarkable feature is that eggs transferred to KCl during the resistant period continue on to cleavage at least beyond the 32-celled stage. This must mean that by virtue of their having been immersed in KCl early, they have acquired the ability to pass through the late susceptible period.

Another feature of interest is the speed of the KCl effect during the susceptible periods. Thus, in the early susceptible period, which is of short duration, further development immediately stopped. Also, during the late susceptible period when the eggs are placed in the KCl a minute or two before cleavage only a small percentage cleave and these cleavages are highly irregular.

Fig. 1 shows the per cent. of eggs which underwent cleavage after they had been immersed in KCl at different intervals. The abscissae represent times after insemination, the ordinates, the percentage cleavage.

(*a*) 0.5′ to 1.5′ After Insemination: The time denotes the moment when the eggs first came into contact with the KCl solution. These eggs were examined at various periods of time, and it was found that the immersion in KCl had immediately arrested further development.

In some, the head of a spermatozoon could be seen at the cortex of the egg, with its tail extended straight and motionless outside. Various stages of the lifting of the fertilization membrane were also observed. In some of them the elevation was complete. In regard to the position of the pigment granules the eggs exhibited the characteristic unfertilized state in which the granules lie scattered throughout the interior of the egg. Some of these eggs were continuously observed for half an hour with no sign of motion, either of further entry of the spermatozoon, lifting of the

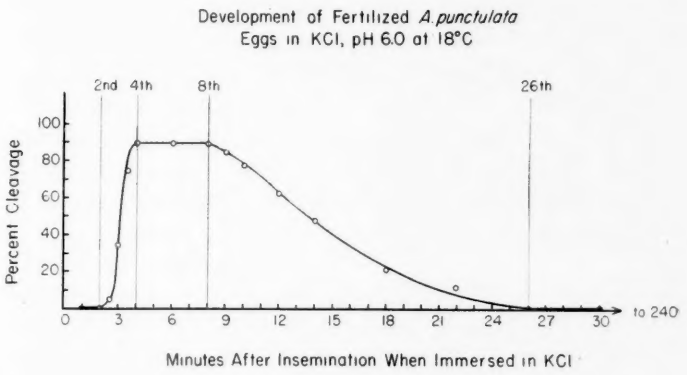

FIG. 1.

membrane, migration of pigment granules or of the egg pronucleus. The eggs were returned at various intervals of time into sea water, and, up to the period of one-half hour, the fertilization process was resumed and the eggs underwent normal first cleavage. The only observable difference between these and eggs which had been continuously in sea water was a delay in time corresponding with the period of sojourn in the KCl solution and the absence in many cases of a fertilization membrane. The ability of these eggs to develop after return to sea water was found to be as follows: After half an hour's sojourn the cleavage was normal and occurred about half an hour after the controls. The hyaline layer (hyaline plasma layer) was sufficient to hold the blastomeres together. After one and one-half hours'

sojourn the viability of the eggs was definitely impaired, and cleavage was abnormal with a slight hyaline layer or in some cases it was absent. After two hours' sojourn the eggs in sea water maintained the unfertilized appearance of their interior and underwent no further development. They continued healthy in appearance as long as do normal unfertilized eggs in sea water. These eggs, however, could not be refertilized.

(*b*) 2.0′ to 3.5′ After Insemination: Eggs immersed in KCl 2.0′–3.5′ after insemination lost their fertilization membranes, although many continued development and cleaved to a variable degree. Those immersed at 2.0′–2.5′ cleaved in small percentages, while those immersed at 3.0′–3.5′ cleaved in large percentages, but mostly not beyond the 2-celled stage. The eggs which did not cleave either remained in an apparently unfertilized state or developed only to the streak or amphiaster stage. A small per cent. may develop as far as the 32- or 64-celled stage, the cells being non-coherent.

(*c*) 2.0′–4.0′ to 8′–13′ After Insemination (Resistant Period): All these develop in high percentages regularly (95 to 98 per cent.) through the 64–128-celled stages. The pigment granules remained intact in their usual position at the periphery of the egg and did not disappear throughout subsequent cleavages. The formation of the typical sperm aster and the subsequent development of the amphiaster occurred as in the normal egg.

Those immersed at 4′ lost the fertilization membrane and showed no sign of a hyaline layer. The blastomeres either fell apart or remained connected each by a long slender intercellular bridge. They appeared as colonies of loosely aggregated cells. Those immersed from 4.5′–8′ maintained their fertilization membrane and the developing blastomeres remained together but not coherent, and there was no sign of a hyaline layer. These blastomeres lay at random. Some of these at the 64–128-celled stage were transferred to sea water. The spaces among them exhibited a fine granular precipitate, presumably of precipitated hyaline layer material, which had been retained in the dispersed state within

the fertilization membrane. Many of the aggregates developed into typical blastulas which continued their development into swimming embryos. On being slightly torn with micro-needles, the embryos readily fell apart, indicating that whatever hyaline material had formed in sea water was very weak and fragile. Eggs immersed in KCl during this period are designated KCl-*resistant* eggs, since they are able to develop in KCl through all further stages, at least up to the 64–128-celled stage. On return of the KCl eggs to sea water at any time up to six hours after immersion, the eggs exhibited complete recovery and continued normal development. The sojourn in KCl had not caused any permanent damage.

(*d*) 8′–13′ to 15′–26′ After Insemination: These eggs underwent development to a variable degree. Those immersed earliest cleave in large percentages, whereas those immersed later cleave in smaller percentages. The eggs remaining single may or may not develop through to later stages. These eggs which cleave may develop only as far as the two-celled stage.

(*e*) 15′–26′ and Later After Insemination (Susceptible Period): Immediately upon immersion in KCl further development ceased. This was found to be true for the eggs immersed at frequent intervals of time up to 240′ after insemination and at the 32- to 64-cell stage. No rhythm of resistance and susceptibility could be detected during successive cleavage stages. The immediacy of the cessation was demonstrated by the fact that the eggs just before cleavage exhibiting karyokinetic lengthening were stopped at this stage by the immersion.

The astral radiations of eggs in the monaster or amphiaster stage disappear within 10′ after immersion. Most of the pigment vacuoles, all of which lay in the periphery, disappeared, leaving the eggs relatively colorless. The hyaline lake enclosing the amphi-nucleus of the monaster stage persisted until cytolysis eventually occurred 2 to 3 hours later. Complete recovery was possible if the eggs were returned to sea water within 30′ and the delay in cleavage time approximated the length of sojourn of the eggs in KCl.

Of these eggs which were immersed at the height of the sperm aster and the amphiaster of the first cleavage, the cleavage time was definitely prolonged over that of the eggs which had been placed in KCl during the interkinetic stages.

The recoverability of eggs immersed in KCl for a longer period than 30′ was characterized by the exhibition of a periodic fluctuation. The relation of this fluctuation to the particular stage when the eggs were transferred to KCl is indicated as follows: When the eggs were placed in KCl at the height of sperm aster development, the recoverability in sea water was very low, as indicated by an extremely small percentage which cleaved. Among these, where cleavage did occur, the time was greatly prolonged beyond the length of the previous sojourn in KCl. When the eggs were placed in KCl during the streak stage, the recoverability was high. In these cases the delay in cleavage time more nearly approached the length of previous sojourn in KCl. Where the eggs were immersed in KCl solution at the height of development of the amphiaster, just prior to cleavage, the recoverability again fell, to rise during the interkinetic stage between first and second cleavage. In all these, the delay in cleavage time was over that which would be expected as a reflection of the length of sojourn in KCl.

The difference between the early period from 0′–2.0′ after insemination and the susceptible period should be emphasized. Eggs immersed in the KCl solution within the first 2.0′ maintain a healthy appearance long after eggs immersed during the susceptible phase have undergone complete cytolysis. The block to development during the early period is probably due to an effect of the KCl on the spermatozoon, whereas during the susceptible period the block is exerted directly upon the egg.

2. Effect of K Salts Other than the Chloride on the Fertilized Eggs of *A. punctulata*

The salts used were sulfate, formate, acetate, citrate, oxalate and tartrate. These were all adjusted to pH 7.0 without buffer. The salt solutions were isotonic with sea

water, and no change in diameter of spherical eggs occurred after immersion.

The sulfate, citrate, oxalate and tartrate all revealed resistant periods similar to the chloride, and development during the resistant phase was superior to that in KCl. The formate and acetate were highly toxic and inhibited development at all periods.

3. Effect of KCl on the Eggs of *Strongylocentrotus purpuratus* and *S. franciscanus*

Essentially the same phenomena were observed in these eggs (Fig. 2). However, an important difference between these eggs and those of *A. punctulata* is that rhythmic periods of resistance are observed prior to both first and second cleavages.

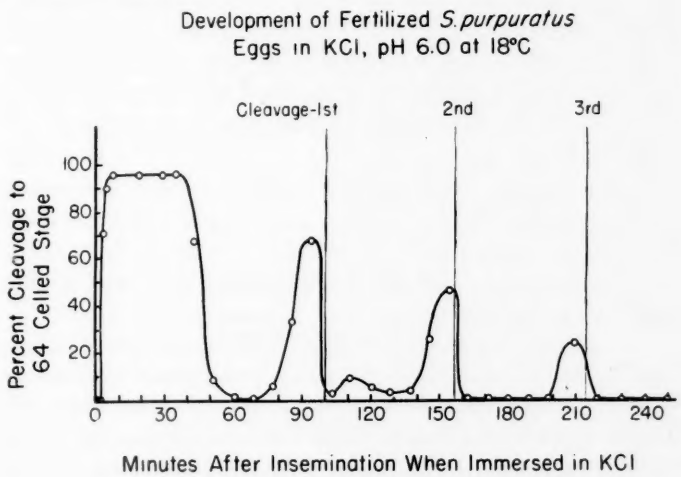

Fig. 2.

4. Salts Which Counteract and Salts Which Sustain the Specific KCl Resistance of Arbacia Eggs

(*a*) Sea Water: Eggs inseminated in sea water were transferred within five minutes into a solution of KCl, pH 6.0 at 18° C. A quantity of the eggs was removed 25′ later

(30′ after insemination) exposed to sea water for 1′ and returned to KCl. These eggs failed to develop, indicating that a brief exposure to sea water of eggs which had been in KCl, will, in so far as susceptibility is concerned, make them react as if they had been continuously in sea water. It is to be noted that the time of 30′ when they were transferred to KCl is the time when the eggs would be expected to be susceptible to KCl.

(*b*) Individual Salts of Sea Water: The next step was to ascertain which of the salts present in sea water are responsible for this effect. In order to arrange this experiment, it was necessary first to ascertain the effect of continuous immersion in each of the salts to be tested.

Accordingly there were prepared isotonic solutions, at pH 6.0 and pH 8.0, of NaCl, Na_2SO_4, $MgCl_2$ and $CaCl_2$. Eggs inseminated in sea water were transferred 25′ later into each of the above salt solutions. Eggs in the NaCl, Na_2SO_4 and $MgCl_2$ at pH 6.0 and 8.0 failed to undergo cleavage. Eggs exposed to $CaCl_2$ at pH 6.0 also failed to cleave, but those in $CaCl_2$ at pH 8.0 underwent cleavage at least up to the 4-celled stage.

The retransfer experiment with KCl was now repeated. Eggs were placed in KCl 5′ after insemination, left there for 25′ and then exposed for 2′ and 10′ to each of the sodium and magnesium salt solutions after which they were brought back to the KCl solution.

(1) NaCl, Na_2SO_4 and $MgCl_2$: The eggs exposed to the NaCl, Na_2SO_4 and $MgCl_2$ at pH 6.0 and 8.0 continued to develop in the KCl solution with the exception that the longer sojourn of 10′ resulted in the non-development of a certain percentage of the eggs. The probability that this is due to the toxicity of the longer sojourn was tested in the following experiment.

The length of the sojourn of the eggs in the above four salt solutions was varied and some of the eggs returned to sea water at the same time that the remainder were being placed back in the KCl solution. It was found that the percentage of eggs which now failed to develop in the KCl was the same as in the sea water. It was therefore con-

cluded that whatever failure of development occurred in the KCl solution was due to the toxic effect of the sojourn in Na and Mg salt solutions. Tests were also made with various mixtures of NaCl and $MgCl_2$ at pH 6.0 and 8.0. No difference was found from the action of these salts used individually.

The fact that the eggs which were not affected by immersion in these salt solutions continued to develop on retransfer to KCl indicated that none of these salts could be responsible for the action of the sea water on the KCl resistance.

(2) $CaCl_2$: The remaining salt to be tested was $CaCl_2$. It was found that a transfer at 25 minutes of the KCl-resistant eggs to the $CaCl_2$ (either pH 6.0 or 8.0) for as short a time as 1′ produced the same effect as the sea water in stopping further development upon return of the eggs to KCl. The eggs left continuously in the pure $CaCl_2$ developed through the 8-cell stage.

Experiments were then made to ascertain the minimum concentration of $CaCl_2$ which would induce this specific effect. The calcium was diluted with isotonic NaCl solution. The specific action of the $CaCl_2$ was evident in dilutions down to 0.002 M $CaCl_2$ in .52 M NaCl. With further dilutions, *e.g.*, .001 and 0.005 M $CaCl_2$, the effect of the mixture increasingly approximated that of NaCl alone. The eggs may be placed at any time in a salt solution at pH 8.0 of .51 M NaCl and .01 M $CaCl_2$, in the same proportion as occurs in sea water, and normal cleavage follows. However, the development does not continue as well as in sea water in which the other essential ion is K and possibly Mg. The concentration of $CaCl_2$ may be progressively decreased down to .002 M, without significantly impairing development. Below this concentration the toxicity increases, until at .0005 M $CaCl_2$ the effect approximates that of pure isotonic NaCl.

Dilutions were also made of the $CaCl_2$ with .53 M KCl. In these mixtures the specific action of the Ca was lost only at concentrations below 0.08 M $CaCl_2$.

(*c*) Other Salts—RbCl, LiCl, $BaCl_2$ and $SrCl_2$: Analo-

gous experiments were made using Rb, Li and Ba. These gave results similar to the results with Na and Mg. Experiments using Sr gave results similar to those of Ca, except that the Sr ion was less effective in destroying KCl resistivity.

From these experiments the conclusion may be drawn that the ability of Arbacia eggs to develop in pure KCl when immersed early is due to the lack of the calcium ion. The presence of calcium in sea water is responsible for the development of susceptibility to the isotonic KCl.

5. The Exchange Rate of K in the Eggs of *S. purpuratus* and *A. punctulata* Immersed in Sea Water

We next became interested in finding what relation the observed periods of resistance and susceptibility to KCl might have to differences in ion exchange during different stages of development. The research was then brought to the laboratory of the late Dr. S. C. Brooks, and radioactive ions were used to measure ion exchange rates. Our objective was to find whether differences in ion exchange could be detected in the eggs at various stages of development.

A dilute (.2 per cent.) suspension of unfertilized eggs was prepared, and an equal quantity added to each of two beakers. Carrier free K^{42} was then added and, simultaneously, the eggs in one beaker were inseminated, while the eggs in the other beaker were left unfertilized. The uptake of K^{42} was then followed by taking frequent samples for radioactivity measurements. Correction was made in each sample for the amount of radioactivity present in the suspension fluid surrounding the eggs. Samples of eggs and suspension fluid were also taken for determinations of the K content. The results for K^{42} appear in Fig. 3. The ordinates represent counts per minute per ml. of eggs, and the abscissae represent time in minutes after addition of K^{42}. In the case of the fertilized eggs this is the same as time in minutes after insemination. The unbroken and interrupted lines represent, respectively, the uptake of K^{42} by the fertilized and unfertilized eggs. The dot-dash line shows the

level of activity in the suspension fluid per ml. The crosses U, V and W, and X, Y and Z indicate the times when samples were taken for analyses of the total K content. The K content in the fertilized eggs remained constant from the time of insemination, at U, to 1000′, at Y. The K content of the unfertilized eggs remained constant through 500′ at

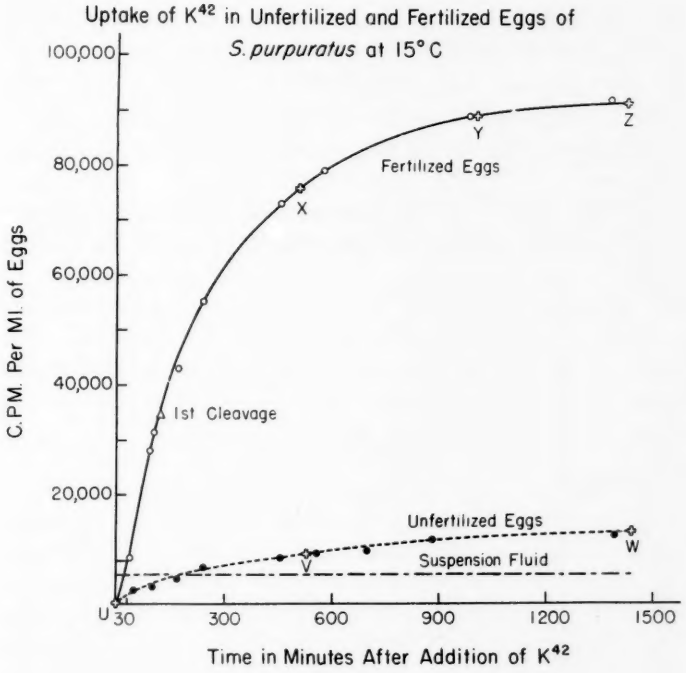

FIG. 3.

V, but thereafter a fall was observed at W. The rate of uptake of K^{42} by the unfertilized eggs is very slow, while in the fertilized eggs the rate is very much more rapid. It is important to note that a marked increase in uptake of K^{42} by the fertilized eggs is observed long before first cleavage (see Fig. 3). However, the increased uptake of K^{42} does not reach a maximum until 40′–60′ after insemination. Fig. 3 is drawn on too large a scale to show this.

Similar experiments were performed using the eggs of *A. punctulata,* and the same results were obtained.

A study of the rate of exchange of K^{42} in eggs immersed in sea water reveals, therefore, that the exchange rate is very slow in the unfertilized egg. After fertilization, the exchange rate becomes very rapid. This increase does not become maximal until a definite period after fertilization.

6. The Exchange Rate of K and Na in the Eggs of *A. punctulata* Immersed in KCl

The next step was to determine the exchange rate of K and Na in pure KCl of the unfertilized eggs and of eggs in the resistant and susceptible phases of development. Radio-

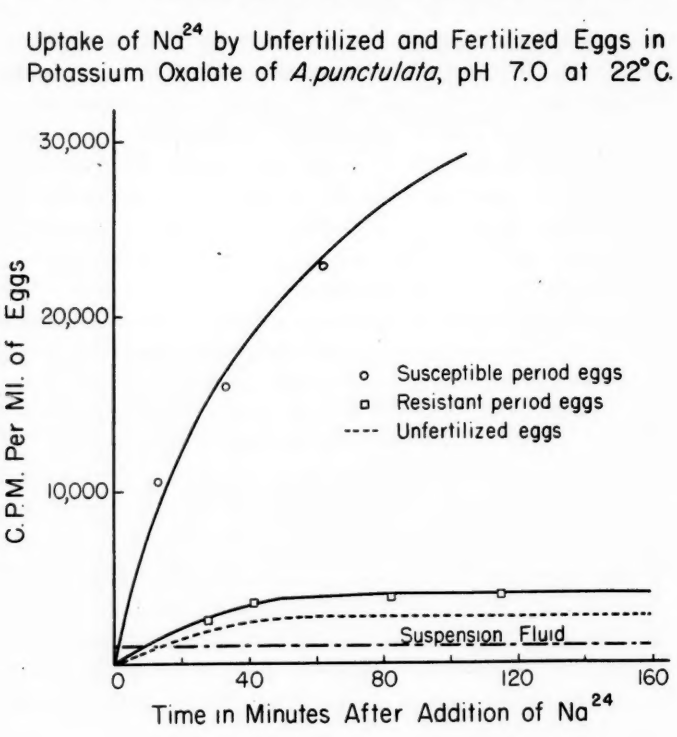

Fig. 4.

active K^{42} was added to a suspension of unfertilized eggs in sea water. After the eggs had taken up sufficient K^{42}, a portion of them was inseminated. The eggs containing the K^{42} were then placed in isotonic KCl and the rate of loss measured. It was found that the rate of loss of K^{42} in unfertilized eggs and in eggs immersed in KCl within 2.0′ after insemination was relatively slow, and somewhat more rapid in the resistant period eggs. In the susceptible period eggs, however, the loss was very rapid, all of the K^{42} having diffused out within 1′ to 2′ after immersion of the eggs in KCl.

The experiment was also performed using Na^{24}. Minute quantities of NaCl containing Na^{24} of high specific activity were added to suspensions of eggs in pure KCl. One suspension was of unfertilized eggs, the second of fertilized eggs in the resistant phase, and the third of fertilized eggs in the late susceptible phase. The rate of uptake of Na^{24} was measured in each of the three suspensions (see Fig. 4). The uptake of Na^{24} by the unfertilized eggs and the resistant, fertilized eggs was negligible, whereas a rapid uptake of Na^{24} was observed in the susceptible period eggs.

7. Conclusion

The experiments with radioactive ions indicate that when eggs are transferred from sea water to KCl, the relative differences in rates of ion exchange observed in sea water are maintained. The extraordinary feature is that fertilized eggs immersed in pure KCl during the resistant period have a low rate of ion exchange in spite of continued development in the solution.

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Discussion

Chairman Engle: It must be wonderful where you can work with organisms and a bucketful at very little cost. I am amazed at the uniform precision with which you can make observations. Dr. Chambers's paper is open for discussion.

Dr. Zeuthen: Have you any opinion whether the potassium ion is in some fixed combination in the cytoplasm or not? In the former case the exchange would measure the rate at which ions are released from such fixed combinations. In the latter case the exchange rate must depend on the permeability of the plasma membrane.

Dr. Chambers: That is a highly debatable point. Our impression is that ion exchange rates are determined by the forces which attract or hold the ions to the proteins and other structures of the cell. It is more a question of the ability of the ions to get away from these forces than the ease with which they are able to cross the membrane.

Dr. Moore: There is a point which I think might be looked out for, and that is the potassium development which Frank R. Lillie showed years ago. He obtained gastrulae without cell division in sea water containing an excess of potassium. I thought possibly you might have observed the internal changes without cell division.

Dr. Chambers: In our experiments the susceptible and resistant phases were concerned with eggs exposed to KCl completely free of sea water. In the susceptible period the cessation of development was of the cell as a whole including its nucleus. Dr. Lillie's findings seem to have no relation to ours.

Dr. Moore: Do you relate your findings to the other rhythmical changes that have been noted?

Dr. Chambers: The significant feature of this work is that by virtue of immersing the eggs in KCl at an early stage of development, they now have the ability to pass through later highly susceptible periods. This phenomenon bears no apparent relationship to the earlier work on rhythmicity. Those experiments mentioned in the body of

the paper in which the development of Arbacia eggs in sea water was observed after a period of immersion in KCl at various stages of development, however, are comparable. The Arbacia egg is susceptible, upon transfer from sea water to KCl, during periods of marked astral development (monaster, amphiaster). It is resistant for a short period immediately after insemination and during the resting phases of development. In the experiments with KCl, except for two short transitional periods, development in the KCl either continued unimpeded or it was immediately stopped. Furthermore, at no time did the KCl appreciably alter the volume of the eggs. These results are similar to those of Lyon (1902) who placed eggs in sea water to which KCN was added, and in sea water at 0° C. at various intervals of time after insemination. The experiments with KCl are directly comparable to Lyon's experiments, since in both cases the volume of the eggs was unaltered, and development was immediately stopped by the immersion.

The increase in susceptibility of eggs immediately prior to and during cleavage to hypotonic sea water (R. S. Lillie, Just), hypertonic sea water (Moore), and higher alcohols (Baldwin), and ether (Spaulding) has been demonstrated. In experiments of this type, which involve a considerable alteration in cell volume, such physical factors as the consistency of the protoplasm and its various components, and the extraneous coats undoubtedly play an important role in determining the resistance of the egg.

DR. TYLER: When the eggs develop in pure KCl in the early stages, is the development relatively quite normal? And is the hyaline plasma layer formed?

DR. CHAMBERS: The development of the eggs in the KCl is quite normal for at least the first three cleavages, with the one exception that the KCl eggs apparently retain all their pigment vacuoles. In normal development in sea water, there is a progressive diminution of the pigment vacuoles in the cortex. The hyaline layer is not visible in eggs developing in KCl. If the fertilization membrane has been removed before immersing the eggs in KCl, the com-

plete nakedness of the surface of the egg can be demonstrated by the oil coalescency method using a micropipette. However, if eggs which retain their fertilization membrane are allowed to develop in KCl, and are then replaced in sea water, a fine, diffuse granular precipitate forms between the surface of the egg and the fertilization membrane. This precipitate undoubtedly is due to the interaction of calcium with the hyaline plasma layer material, which had been formed in the KCl, but had remained in a soluble state within the confines of the fertilization membrane.

DR. TYLER: Is fertilization possible in KCl? I think you have tried that.

DR. CHAMBERS: Spermatozoa are immediately immobilized in KCl at pH 6.0, and fertilization of the eggs is impossible. I am interested in what you think about the initial susceptible phase to KCl. Do you think this represents the inhibitory action of the KCl directly on the sperm?

DR. TYLER: It wouldn't take more than 15 to 20 seconds for the sperm to enter.

DR. CHAMBERS: Yet the initial susceptible phase lasts two minutes. During this phase the sperm remains at the surface of the egg.

DR. TYLER: Several people, including myself, now think that the establishment of the block to polyspermy may be a rather slow affair, and that various sperms start into the egg, but after a short time all but one of them are repelled. There is some recent evidence concerning this which Dr. Rothschild has accumulated in studies of the cortical changes upon fertilization. In contrast to earlier workers he finds that these changes are relatively slow.

METABOLISM OF SEA URCHIN SPERM[1,2]

DR. JOHN D. SPIKES

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THE physiology of fertilization, at least for convenience, may be investigated from two aspects with regard to the participation of the spermatozoon. First, the process by which the sperm reaches the egg must be studied. In most species of animals the sperm is motile; thus it becomes necessary to determine how the energy for motility is obtained. This involves a study of the metabolism of the sperm cell. The second aspect concerns the mechanism by which the sperm attaches to and penetrates into the egg after reaching its surface. It is probable that sperm motility is not necessary for this second process. The metabolism of the sperm of certain vertebrates, especially mammals, has been extensively studied, but little is known of the interactions between the sperm and eggs of vertebrates. Conversely, remarkably little work has been published on the sperm metabolism of invertebrates, while extensive work has been done on the interactions between the sperm and eggs of certain invertebrates.

Materials used in this work were sperm of the Pacific coast sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus pictus*. The results obtained with the two species were essentially the same, although certain quantitative differences were observed. The Warburg technique was used for measuring oxygen consumption and carbon dioxide production.

[1] This work was carried out at the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena.

[2] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.

Mann (1946) showed that the seminal fluid of most vertebrates contained fructose and that this was the normal substrate of the sperm. Lardy and Phillips (1941) showed that bull sperm deprived of external substrate were capable of oxidatively metabolizing endogenous phospholipid. It was thus of interest to determine the normal substrate of sea urchin sperm, since sea urchin sperm leads a somewhat different life from mammalian sperm. It is shed free into the sea water and is thus highly diluted. As a result, even if there were a substrate present in the so-called seminal fluid it would not be of much use to the sperm. The sea urchin sperm therefore has to carry its own total supply of substrate.

The respiratory quotient of sea urchin sperm is approximately one. This indicates that they are metabolizing carbohydrate. A better way to determine the substrate utilized is to perform analyses on the sperm for a number of possible substrates before and after aging the sperm under controlled conditions. The results of a typical experiment of this kind are shown in Table 1. As may be seen,

TABLE 1

CHEMICAL CHANGES IN STRONGYLOCENTROTUS SPERM DURING 12 HOURS OF AGING AT 19 DEGREES CENTIGRADE

(Expressed in mg. per gm. dry wt. of sperm)

Constituent	Before aging	After aging
Reducing value, total	30.6	9.3
Reducing value, TCA sol.	1.4	0.7
Reducing value, glycogen-like material	29.0	8.5
Total lipid	104	107
Neutral lipid	48	49
Phospholipid	52	54

the only constituent showing a marked change with aging is the reducing value of the glycogen-like fraction. This agrees with the value of the respiratory quotient. It would seem therefore that the normal substrate of the sperm is a glycogen-like substance.

The next step was to determine whether or not the sperm could utilize added substrate. It has been shown that mammalian sperm can utilize added substrate. Unfortunately none of the compounds tested, which included a wide

variety of carbohydrates and the usual intermediate compounds in carbohydrate and fat breakdown, had any effect on the oxygen uptake of the sperm. Certain materials, such as glucose, fructose, sorbose, etc., were capable of prolonging sperm life by as much as 300 per cent. however. This would indicate that these compounds were able to penetrate only very slowly, and thus that their effect could not be evident in the short time involved in a Warburg determination.

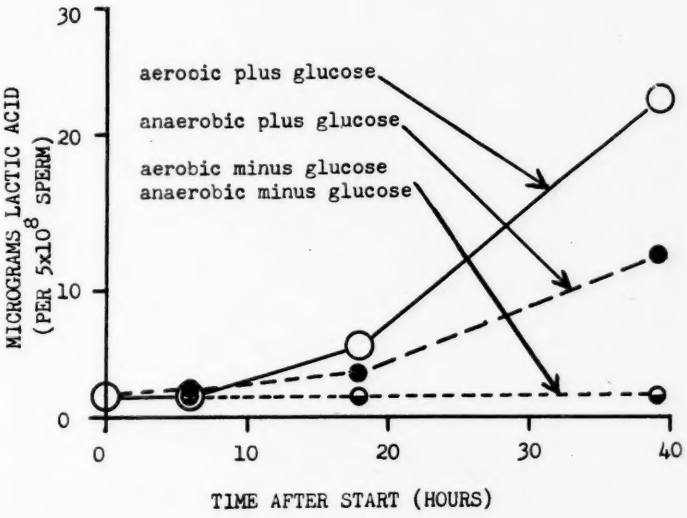

FIG. 1. Lactate production of sea urchin sperm.

The intermediate pathways through which the sperm break down carbohydrate were next investigated. While mammalian sperm appear to be preferentially glycolytic in their metabolism even under aerobic conditions, it was found that sea urchin sperm can produce only small quantities of lactic acid from carbohydrate. Fig. 1 shows the lactate production by sea urchin sperm under various conditions. It will be noted that lactate production is greater under aerobic conditions, and is markedly increased by the addition of glucose. Most tissues show an increase in the

rate of glycolysis under anaerobic conditions. None of the substrates tested were capable of maintaining sperm motility under anaerobic conditions. This is in contrast to mammalian sperm, which survive longer under anaerobic conditions than in the presence of oxygen.

Fig. 2 shows the effect of certain inhibitors on the rate of lactate production by sperm under aerobic conditions.

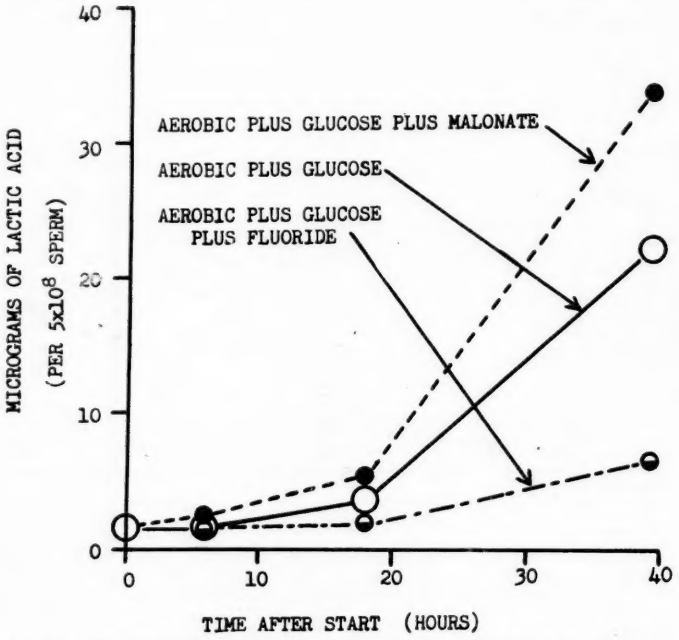

FIG. 2. Lactate production of sea urchin sperm in the presence of certain metabolic inhibitors.

Added fluoride inhibits the formation of lactate in the presence of glucose. Malonate on the other hand increases the rate of formation of lactate.

It was not found possible to demonstrate the presence of the enzyme phosphorylase in a brei of sea urchin sperm. It might be that the system is inactivated in the preparation of the brei. The use of intact sperm gave negative results

also, but this would be expected because of the relative impermeability of the sperm. A phenomenon was observed, however, which might be related to the process of phosphorylation. If glycogen or glucose and inorganic phosphate were added to a sperm brei it was observed that the inorganic phosphate concentration of the system rapidly decreased. This might indicate that these materials were being phosphorylated inasmuch as this rapid decrease was not observed in the absence of added glycogen or glucose.

One of the stages in the breakdown of carbohydrate according to the classical schemes of glycolysis is the formation of fructose diphosphate. Fig. 3 shows the formation

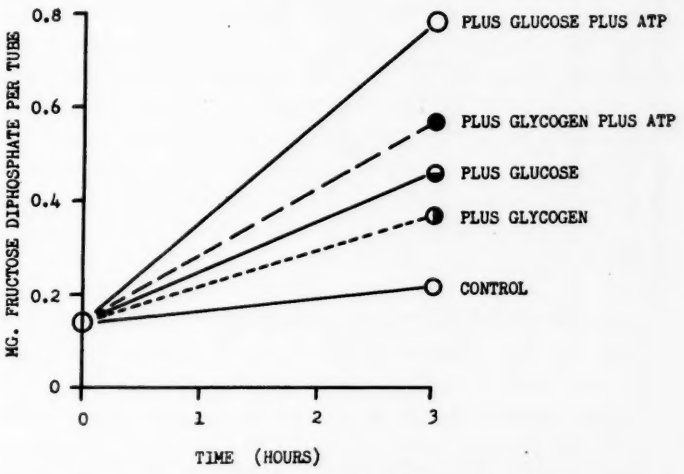

FIG. 3. Formation of fructose diphosphate by sea urchin sperm brei.

of this compound under various conditions in a brei of sea urchin sperm. The curves show that the rate of formation of fructose diphosphate is significantly increased by the addition of glycogen or glucose. The rate of formation is increased further by the presence of ATP. These results indicate that the formation of fructose diphosphate in sea urchin sperm from glucose and glycogen probably occurs in

the same manner as in yeast, mammalian muscle and most of the other tissues which have been studied.

In the classical schemes of glycolysis, fructose diphosphate is converted to triose phosphate. Fig. 4 shows the conversion of fructose diphosphate to triose phosphate by a brei of sea urchin sperm. Calculations show that this conversion occurs at roughly the same rate as that carried on by the isolated nuclei of rat liver cells, the only tissue comparable to sperm for which data were available.

The formation of pyruvate and lactate from triose phos-

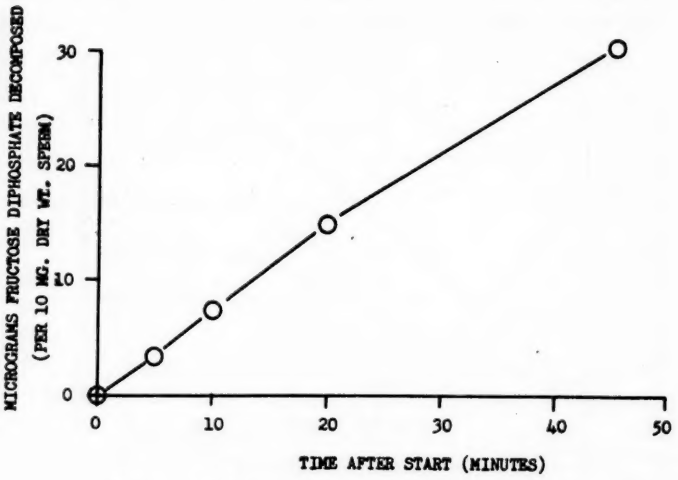

FIG. 4. Aldolase activity of sea urchin sperm brei.

phate was not observed directly. However, the overall conversion of hexose to lactate, and the intermediate conversion of hexose to fructose diphosphate, of fructose diphosphate to triose phosphate and of pyruvate to lactate have been observed. Thus it would appear that sperm could carry out the conversion of triose phosphate to pyruvate and lactate. Further, it has been shown that the rate of lactate formation from hexose was inhibited by the addition of fluoride. It is known that fluoride somewhat specifically inhibits a reaction in the series of reactions by which triose phosphate

is converted to pyruvate and lactate. It was also observed that the inhibition of the oxygen uptake of sea urchin sperm by added fluoride could not be reversed by the addition of hexose or of any other compounds above 2-phosphoglyceric acid, while the inhibition could be reversed by compounds below 2-phosphoglyceric acid such as pyruvate. Thus it may be concluded that the reactions involved in the conversion of triose phosphate to pyruvate and lactate in sea urchin sperm are essentially the same as those occurring in muscle and in yeast.

The mechanisms involved in the oxidation of three carbon compounds (pyruvate) are not nearly as well understood as are those involved in glycolysis. Probably the best mechanism suggested to date for this process is that of Krebs and his coworkers (1937–1943) which is commonly known as the Krebs tricarboxylic acid cycle. According to this suggestion, pyruvate and oxalacetate are condensed, and the product is oxidized and decarboxylated to yield cis-aconitic acid which adds water to form isocitrate. This is oxidized and decarboxylated to yield α-ketoglutarate which in turn is oxidized and decarboxylated to give succinate. The succinate is oxidized to fumarate, and this is converted to malate which is oxidized to oxalacetate.

Addition of intermediates of the Krebs cycle to sea urchin sperm had no effect on the oxygen uptake of the tissue. Further, sperm were only very slightly inhibited by the addition of malonate, a specific inhibitor of succinic dehydrogenase, which is one of the key enzymes of the Krebs cycle. It would appear then that the sperm are relatively impermeable to these compounds as they are to carbohydrates and to the intermediates of glycolysis.

It has been established that tissues generally are more permeable to unionized materials than to ionized. Thus it might be supposed that esters of the intermediates of the Krebs cycle would penetrate into the sperm, be hydrolyzed there, and then be acted upon by the enzymes involved in the cycle. It was found however, that instead of increasing the rate of oxygen consumption of the sperm, the

esters acted as inhibitors. Ethyl citrate, ethyl succinate, ethyl malonate, ethyl fumarate and ethyl malate all gave approximately the same concentration versus inhibition of oxygen uptake curves. This suggested that the esters might

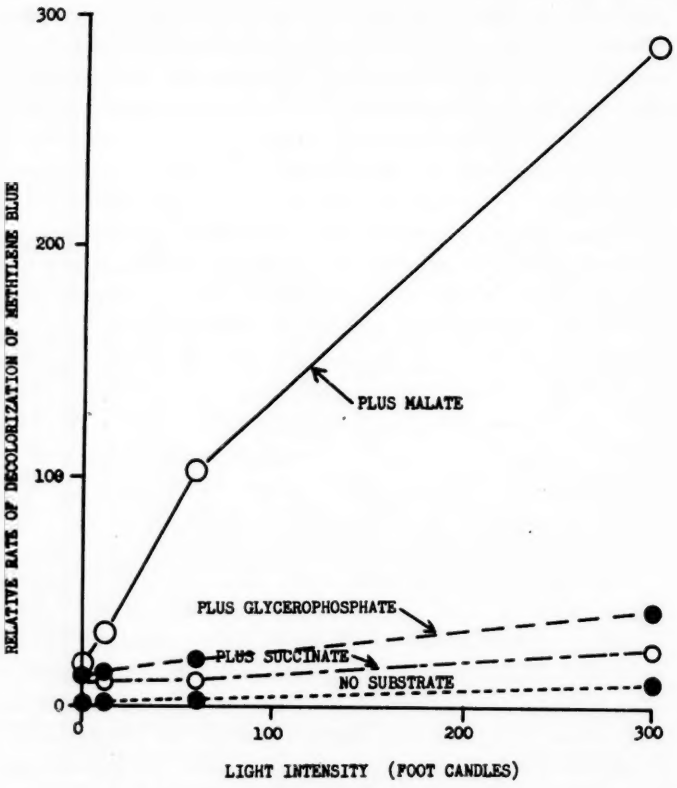

FIG. 5. Effect of white light on the activity of sea urchin sperm dehydrogenases as measured by the Thunberg technique.

be acting as competitive inhibitors of the corresponding salts in a system similar to the Krebs cycle. This possibility was tested for by using in vitro systems of succinic and malic dehydrogenases from the sperm. It was found that the esters acted as highly specific, competitive inhibi-

tors of the corresponding dehydrogenases. It was also shown that sperm breis could interconvert succinate, fumarate and malate. Thus it would appear from the above indirect evidence that some mechanism resembling the Krebs cycle is in operation in sea urchin sperm.

An interesting phenomenon was observed in connection with the work on dehydrogenases. It was found that the

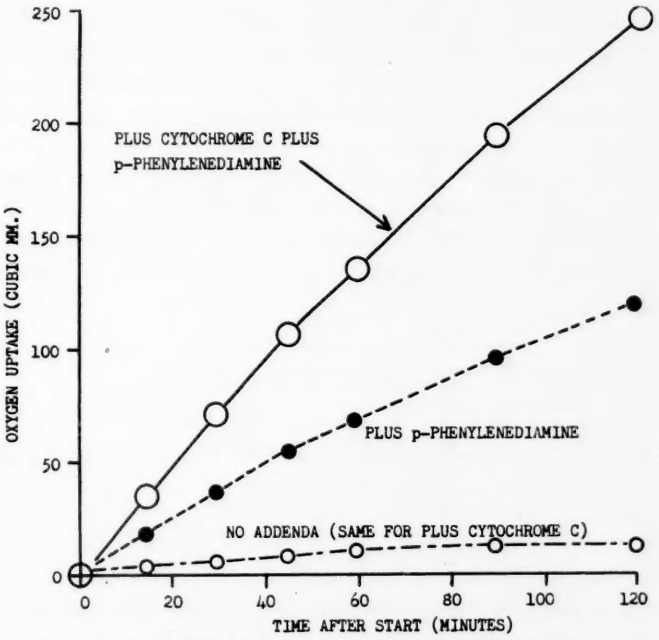

FIG. 6. Cytochrome oxidase activity of sea urchin sperm brei.

apparent activity of the malic dehydrogenase system was markedly accelerated by exposure to white light. This is shown in Fig. 5. It was found further that this effect was produced only by the red region of the spectrum. This may correlate with an observation made by Professor MacGinitie. He observed a number of years ago that the motility of Urechis sperm is greater in light than in the dark.

The terminal oxidase of mammalian sperm has been

shown to be cytochrome oxidase. Sea urchin sperm also contains an active cytochrome oxidase, as is shown in Fig. 6. The curves show that the rate of oxidation of p-phenylenediamine by a sperm brei is markedly accelerated by the addition of cytochrome c. This indicates the presence of cytochrome oxidase in the sperm. Catechol was not oxidized by the sperm.

An adequate explanation of the coupling between the energy produced by metabolism, and the utilization of this energy by cells to perform work has not been described. It would appear that in most tissues energy can be transferred by means of so-called high energy bonds, such as the two terminal phosphate bonds in adenosine triphosphate (ATP). It was found that sea urchin sperm contain relatively large quantities of a barium insoluble, acid labile phosphate ester which might be ATP. Further it was found that the sperm contain a highly specific ATP-ase. This is shown in Table 2.

TABLE 2

PHOSPHATASE ACTIVITY OF STRONGYLOCENTROTUS SPERM

(Expressed in terms of relative change in inorganic phosphate concentration over zero time)

Substrate	15 min.	30 min.	60 min.
None	0.4	4.8	9.2
Glucose-1-phosphate	−2.8	−2.0	−3.2
Fructose-1, 6-diphosphate	0	0	3.6
Na glycerophosphate (52% a)	0	1.6	4.0
ATP (sodium salt)	27.2	54.8	80.4

As the data show, sperm brei has a very active ATP-ase, but apparently lacks phosphatases for the other phosphate esters.

In connection with sea urchin sperm it was of interest to observe the effect on sperm metabolism of the material from the surface of the eggs (fertilizin) which specifically agglutinates homologous sperm. It has been reported for some species of sea urchins that fertilizin increases the metabolic rate of the homologous sperm. However, with the species used for this work it was found that fertilizin inhibited the rate of oxygen uptake. It has been suggested that the agglutination per se of the sperm might cause this

decrease in rate of oxygen uptake. This possibility could be tested for because, as Tyler (1941) has shown, fertilizin can be converted to a form (univalent) in which it is still capable of specifically combining with the sperm, but can no longer produce agglutination. This can be done, for example, by irradiating the fertilizin with ultraviolet light. Fig. 7 shows that fertilizin in the univalent form, which does not produce agglutination, no longer inhibits the oxygen uptake of the sperm.

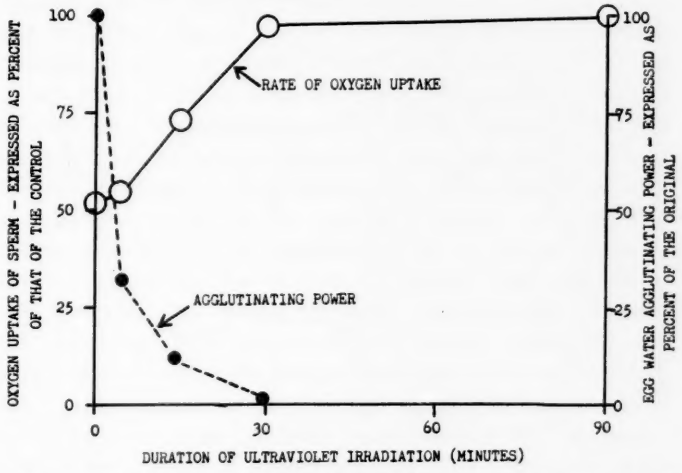

FIG. 7. Effect of fertilizin irradiated with ultraviolet light on the rate of oxygen uptake of homologous sea urchin sperm.

It was of interest to examine the relation between the metabolism of sperm and its ability to fertilize. The fertilizing power of sperm may be determined by making serial dilutions of the sperm in sea water, and adding a known number of eggs to a constant volume of each sperm dilution. The per cent. of eggs fertilized in the various dilutions can be determined, and from these data the concentration of sperm necessary to give 50 per cent. fertilization can be calculated. This figure may be used as an indication of the fertilizing power of a sperm suspension.

As was mentioned earlier, the presence of certain carbo-

hydrates prolongs the life of sea urchin sperm. It can further be shown that the ability to fertilize is maintained for longer periods in the presence of these substrates. In connection with the study of sperm metabolism it was customary to observe the degree of motility and the fertilizing power of the sperm at the end of each Warburg determination. This resulted in three types of data for comparison in each treatment of the sperm: relative oxygen uptake, degree of motility and fertilizing power. The results obtained with a large number of metabolic inhibitors indicated in general that the rate of oxygen uptake of the sperm, the motility and the fertilizing power were directly correlated. Thus it would seem that in these experiments the fertilizing power of a sperm suspension is proportional to its motility (and rate of oxygen uptake). This relationship is apparently found generally. With farm animals, for example, the rate of respiration of the sperm is used as a practical measure of sperm viability and fertilizing capacity.

Motility and concurrent rate of oxygen uptake are not the only criteria of the fertilizing power of a sperm suspension, however. It has been shown by Lillie (1913) and by Tyler (1941) that sea urchin sperm which have been agglutinated by homologous fertilizin show a spontaneous reversal of the agglutination after a time. The reversed sperm appear to be just as motile as before they were agglutinated, but they are incapable of fertilizing eggs. Tyler (1941) showed that if sperm were treated with acid sea water, a treatment which is known to partially remove the specific combining material from the surface of the sperm, the fertilizing capacity of the sperm decreased more rapidly than did the rate of oxygen uptake. Tyler concluded from this that part of the decrease in fertilizing power was due to a removal of the surface material and that this material therefore played an essential role in the process of fertilization. Tyler also showed that the specific combining material on the surface of the sperm was protein in nature. This suggested the possibility that the material could be removed by means of a proteolytic enzyme, which

treatment should result in a complete loss of fertilizing power without any particular decrease in the motility or rate of oxygen uptake. The results of such an experiment are shown in Fig. 8. Crystalline chymotrypsin was used for the enzyme.

The curves show clearly that the oxygen uptake of the sperm in the enzyme remained almost the same as that of the control, while the fertilizing power of the sperm in the

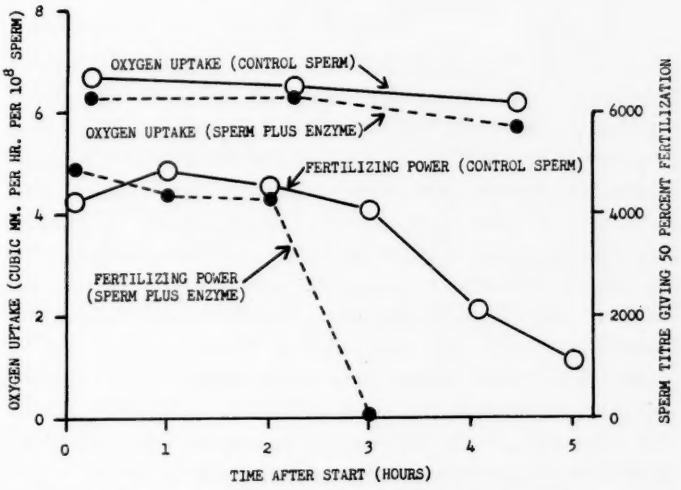

FIG. 8. Effect of crystalline chymotrypsin on the oxygen uptake and fertilizing power of sea urchin sperm.

enzyme fell to zero after a rather short time. It was also observed that the degree of agglutination of the sperm with added fertilizin fell off at about the same rate as the fertilizing power. It would appear, then, that the fertilizing power of sea urchin sperm is a function of two independent factors, (1) the degree of motility of the sperm, which is correlated with its respiratory activity, and (2) the presence of the specific combining substance in a reactive condition on the surface of the sperm.

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Discussion

Chairman Engle: Thank you, Dr. Spikes. The use of the sperm as an object for metabolic studies is something that has gotten under way slowly, but it seems to be gaining in general interest. In the metabolic study on sperm, species differences are observed. It adds a great deal to our knowledge of the sperm cell to observe these differences. Even though species differences occur, one is impressed with the general pattern of uniformity from sea urchins to man. Is there any general discussion of this most interesting topic?

Dr. Horowitz: Were these experiments under aerobic conditions done with living sperm or sperm brei?

Dr. Spikes: Living sperm. I was never very successful in getting lactic acid production with sperm brei.

Dr. Horowitz: Then glucose does get in?

Dr. Spikes: Yes, but very slowly. It apparently doesn't penetrate during the course of an ordinary manometric experiment. If the sperm are in contact with it for 12 to 24 hours some glucose apparently gets in, however.

Dr. Horowitz: Is the lactic acid oxidized by the sperm?

Dr. Spikes: Apparently not.

Dr. Pease: I wondered if you made any effort at all to use different parts of the sperm in such metabolic studies. I suppose it is the middle piece that you are mostly concerned with.

Dr. Spikes: In bull sperm it has been shown that the

cytochrome system is largely localized in the mid-piece and tail. We don't have such data on sea urchin sperm.

DR. PEASE: How about the acrosome? Does it have any appreciable metabolism that you know of in the vertebrate sperm?

DR. SPIKES: There is apparently nothing in the literature on that.

DR. CHAMBERS: Were the phosphatase experiments done with sperm breis?

DR. SPIKES: Yes, very little phosphatase activity was found using intact sperm.

DR. CHAMBERS: Even on ATP?

DR. SPIKES: Yes. There was a slight ATP-ase activity with living sperm, but not as great as with the brei. Apparently the material didn't get in. Dr. Chambers, didn't you find that such materials as radioactive phosphorus penetrated the sperm very slowly?

DR. CHAMBERS: Yes, at about the same rate as into the unfertilized egg.

DR. DELBRUCK: How do you measure motility?

DR. SPIKES: Simply by examining the sperm suspensions under the microscope and making a subjective estimate of their motility. It is possible to classify sperm into about six motility groups by such a method.

CHAIRMAN ENGLE: Is your criteria of motility vibratory action or progression?

DR. SPIKES: It is vibratory action. Motility is a rather difficult point to determine actually. I have never heard of a good method of measuring it.

DR. DELBRUCK: You implied a relationship between motility and oxygen uptake. You had a beautiful curve.

DR. SPIKES: If you were to hand me sperm suspensions I could classify them into six different groups on the basis of motility. I wouldn't care to be any more accurate than that.

DR. DELBRUCK: This enzyme reaction, that was photocatalyzed, it was a dehydrogenation of malic acid?

DR. SPIKES: Yes, as determined by the regular Thunberg method.

DR. DELBRUCK: What sort of an enzyme is that?

DR. SPIKES: In most tissues it is a system involving the dehydrogenase, coenzyme 1, and diaphorase. However, the whole system is not known for sea urchin sperm.

DR. DELBRUCK: You don't know?

DR. SPIKES: Not in this particular system.

DR. DELBRUCK: Is this photocatalytic effect unique?

DR. SPIKES: There is one reference in the literature which indicated that a certain system involving diaphorase and methylene blue was slightly activated by red light. I was never able to see the original paper, however. I thought Dr. Haas might be able to help us out. He has been working with this yellow enzyme system which is affected by light.

DR. DELBRUCK: Is the enzyme destroyed by light?

DR. SPIKES: I really don't know. It might be that some coenzyme system or some inhibitor system is affected by the light rather than the dehydrogenase itself. The malic dehydrogenase system is somewhat different from the other dehydrogenases in sperm. If you lyophilize sperm, you can still get a very nice succinic dehydrogenase activity, but the malic dehydrogenase activity is destroyed.

DR. DELBRUCK: It is destroyed by what?

DR. SPIKES: Lyophilizing.

DR. DELBRUCK: How did you run across this photosensitivity?

DR. SPIKES: I just had a series of tubes set up on the desk, and I noticed that on the side toward the windows the tubes were decolorizing much more rapidly than on the other side.

DR. DELBRUCK: And how were these measurements then done?

DR. SPIKES: I didn't have much equipment or time. I simply worked in a dark room and set up tubes at different distances from the light source. I used an exposure meter

to determine light intensity and then simply observed the rate of decolorization.

DR. DELBRUCK: Decolorization of what?

DR. SPIKES: Methylene blue.

DR. TYLER: You have to add cyanide in this system.

DR. SPIKES: Oh, yes.

DR. DELBRUCK: Is this enzyme well known in the sense that it has been purified or crystallized?

DR. SPIKES: I don't think it has been crystallized.

MR. MACGINITIE: Is there any correlation between the rate of dispersal and motility in the sperm? Would it be possible to put in spots of sperm and determine a better rate of motility by the rate of dispersal?

DR. SPIKES: I spent a lot of time in attempting to devise a good method for objectively determining sperm mobility. That was one of the methods we considered, but it was never tested.

CHAIRMAN ENGLE: If there are no further questions, we will proceed to the remaining paper by our guest from Copenhagen, Dr. Erik Zeuthen, on "Oxygen Consumption during Mitosis; Experiments on Fertilized Eggs of Marine Animals."

OXYGEN CONSUMPTION DURING MITOSIS; EXPERIMENTS ON FERTILIZED EGGS OF MARINE ANIMALS[1]

DR. ERIK ZEUTHEN

HOPKINS MARINE STATION, PACIFIC GROVE, CALIFORNIA, AND UNIVERSITY OF COPENHAGEN

IMMEDIATELY after fertilization the respiratory rate of the egg may undergo considerable changes; in some species it drops, and in other species it increases. After these initial adjustments of the respiratory rate (oxygen uptake per unit of volume) are over, it can be shown that the rate of oxygen uptake is very much the same in a great many different eggs (Whitaker, 1933). Now the fertilized egg starts cleaving, and cleavage follows cleavage at even time intervals, or at time intervals which quite gradually become longer and longer as development proceeds. In this way the egg divides into a great many blastomeres, which may become very small, their aggregate volume all the time being equal to that of the unsegmented egg. Within the single embryo, each mitotic cycle cleaves all blastomeres almost simultaneously, and the same is also true when we consider a great number of eggs. They divide simultaneously, provided the eggs are from the same female, that they are fertilized at the same time, and that they are kept at the same temperature.

In the eggs of the frog, *Rana platyrrhina,* the first three to four division cycles have been studied; the same is so for the egg of the worm, *Urechis caupo* (in the latter case the synchronicity of the divisions within the single embryo is known to stop after the fourth division). The echinoderm

[1] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.

eggs are very well suited for the studies of which I am going to speak, because the first eight to ten cleavages seem to take place in a beautiful rhythm.

It is a characteristic feature of all cleaving eggs that a smooth increase in respiratory rate can be demonstrated during the cleavage period. So, for instance, Lindahl (1939) demonstrated that the respiratory rate of the sea urchin egg increases steadily up to the hatching stage (probably around one thousand cells in the embryo). At about the time when hatching occurs, the respiratory rate remains constant for some time, but another rise in respiratory rate sets in as gastrulation and visible differentiation begin. Up to the stage of hatching, the respiratory rate follows an S-shaped curve. The present paper deals mainly with a fine analysis of this curve in the hope of finding " bumps " corresponding to the mitoses going on. It is important to mention that during this early period the segmentation of the egg into a great many blastomeres appears to be the dominating feature of development. For that reason we dare make the assumption that the gradual change in respiratory rate has some intimate connection with the mitoses going on at the same time.

The first series of experiments (Zeuthen, 1946) to be reported were performed on the frog egg. It was the aim to find out whether the respiratory rate is absolutely uniform or not at the times when this egg undergoes the first, second, and third cleavages. At that time I worked in the Carlsberg Laboratory with Linderstrøm-Lang (1937, 1943) and Holter (1943), who jointly developed the Cartesian diver micro-respirometer. It became apparent, however, that the high sensitivity of the Cartesian diver was not high enough. So the diver was modified to give increased sensitivity for slight respiratory variations. In other respects the diver used was a poor one, but anyway it became evident that the three first mitoses in the frog egg were accompanied by slight respiratory variations (Fig. 2). During the early part of each mitosis there was a slight increase in respiration and the cleavage furrow started digging into the egg during this period (according to Brachet [1934] the

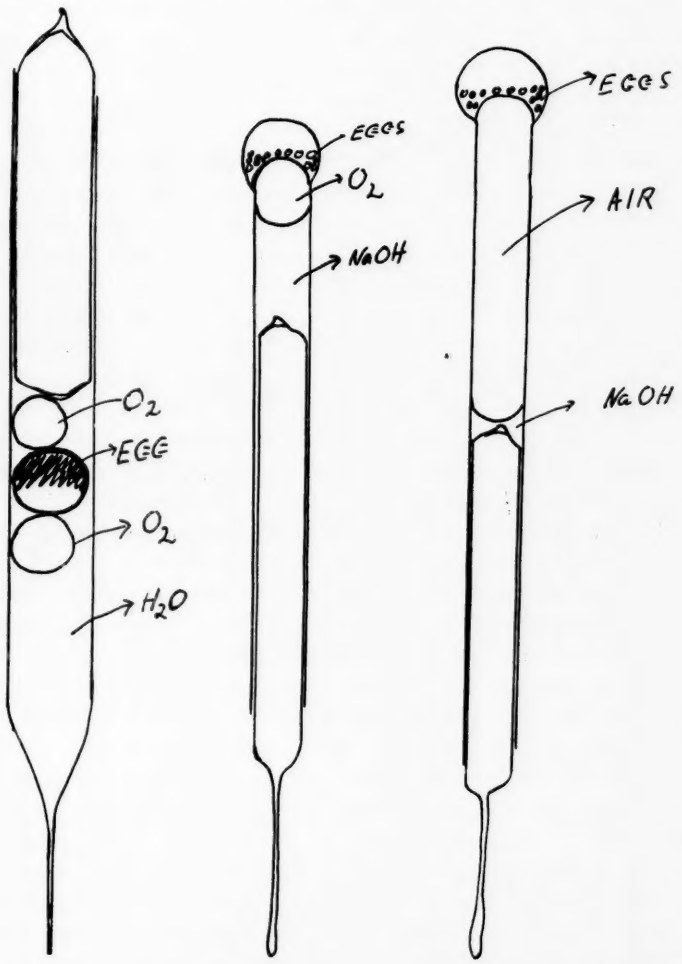

FIG. 1. Three types of Cartesian divers as used for studies on respiration during cell division. *Left:* Diver used in studies on the frog egg (Zeuthen, 1946). The pressure variations in two oxygen bubbles situated close to the egg are followed. No CO_2 absorption, but most of the CO_2 formed is held in solution by the water in the diver. *Middle:* Diver used for studies on the egg of Urechis. *Right:* Diver used in connection with a very sensitive manometer. Used for studies on the eggs of *Strongylocentrotus franciscanus* and *Dendraster excentricus*.

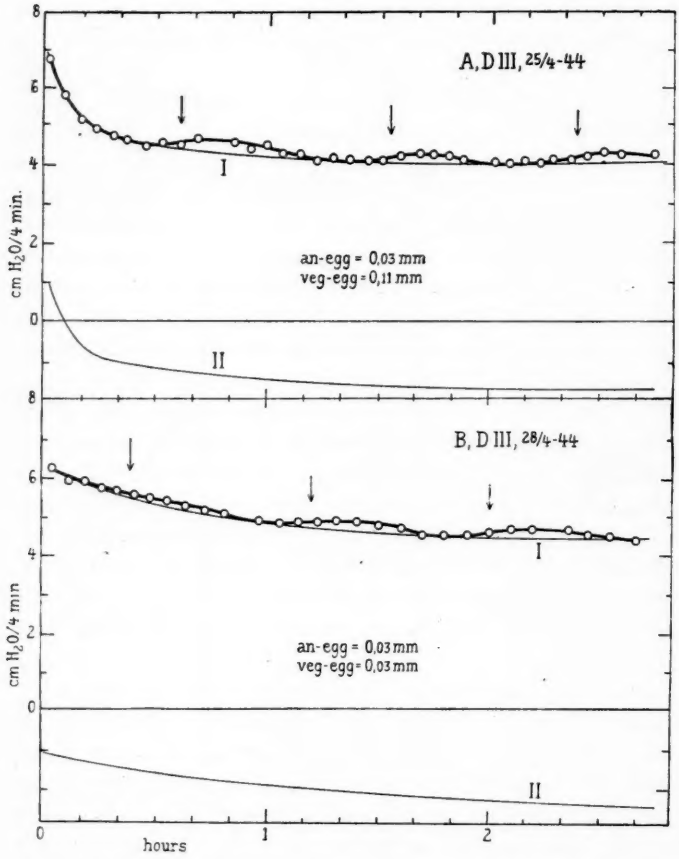

FIG. 2. Two experiments on eggs of the frog, *Rana platyrrhina*. There is one respiratory wave per mitosis. Arrows indicate the time of appearance at the animal pole of the first, second and third division furrows. The initial drop of the curve to the left in the figure is due to technique used (Zeuthen, 1946).

appearance of the cleavage furrow coincides with the prophase). The respiration attains a maximum some ten minutes after the cleavage furrow appears, and after this it starts dropping again. Thus, the respiratory rate fluctuates up and down in the same rhythm as that of the cell divi-

sions going on. The respiratory curve looks like a series of sine waves, one wave per mitosis. Brachet, who used the Fenn technique, had earlier (1934) described another curve for the frog egg having two somewhat larger variations per mitosis. The discrepancies between Brachet's data and mine have never been well explained. As an argument in favor of my results, I mention that I have found essentially constant results in different kinds of eggs (see later).

Linderstrøm-Lang (1946) subjected these results obtained with the diver to a very detailed discussion. The point is that the diver used was specifically adapted for the purpose of detecting a respiratory rhythm, if present at all. This was accomplished at the price of accepting certain drawbacks: In the diver there is no mechanical stirring so that a diffusion path exists between the metabolic centers in the egg and the recording system (two air bubbles situated next to the egg, comp. Fig. 1, left). Moreover, the separation between O_2 and CO_2 in the diver is incomplete, being based on the different solubilities of the two gases in water. These circumstances led Linderstrøm-Lang to the conclusion that the respiratory variations found were indicative of somewhat larger variations in the egg itself. It was considered, however, that the variations in the egg were of the same type as those recorded with the diver, *i.e.*, sine waves with wave heights about 7–11 per cent. of the average respiratory rate of the egg (the height of the recorded waves were 4.5 per cent.). In subsequent experiments on eggs of marine animals full advantage was taken of Linderstrøm-Lang's results: complete CO_2 absorption was secured, the alkaline seal being separated from the droplet containing the eggs. At the same time the diffusion path between the eggs and the air bubble of the diver was reduced to about one-half mm. Due to both circumstances, the *diver should record the respiratory variations in the eggs without either damping or delay.* The divers adopted are shown in Fig. 1, middle and right. One can see from Fig. 1, middle, right, that the diver floats in such a way that the eggs drop down on the interphase between water and air, where they become densely packed. This reduces the diffusion path in

the system to a minimum, fortunately without influencing the development of the eggs. Fig. 3 shows an experiment with several hundred eggs of the worm Urechis. The oxygen uptake of the eggs fluctuates up and down almost like in the frog egg and there is one respiratory wave per mitosis. The cytoplasmic cleavage occurs at the time when the respiration is decreasing, *i.e.*, later than in the frog egg. Cytological studies (by Dr. Irving A. Tittler) indicate that the interphase-prophase coincides with the minimum oxygen uptake. The metaphase-anaphase take place when the respiration is at its height and anaphase-telophase stages are found during the period of decreasing respiration. The difference between the frog egg and other eggs with regard to the appearance of the cytoplasmic cleavage is probably only apparent: In the frog egg the cytoplasm starts dividing during prophase, when the respiration is increasing, but it takes a long time before the furrow embraces the whole egg. In the small marine eggs studied (which all behave like the Urechis egg) the furrow shows up later, when the respiration is again decreasing and the cells are in the telophase. The time correlation between mitotic stage and respiration may well be almost the same in all eggs studied, but the time correlation between these two events and the cytoplasmic division appears to be different in different eggs, depending perhaps on the amount of cytoplasm to be cleaved. If there is much cytoplasm the cleavage takes a long time, and it starts early in the mitotic cycle (frog egg). In Fig. 3 the dotted line indicates the oxygen pressure in the air bubble. The respiration is independent of the oxygen pressure as it drops from 21 per cent. to 6–7 per cent. of an atmosphere. At oxygen pressures lower than this the respiration is no longer independent of the oxygen pressure. Experiments in pure oxygen result in the same type of curve as that of Fig. 3. Apparently, within wide limits the respiratory waves are independent of the oxygen pressure, which, of course, was to be expected. Experiments have also been run with Urechis eggs at different temperatures. As far as I can judge, the wave phenomenon is the same at

all temperatures within which normal development is possible (10–24° C.). Compare Fig. 7.

I mentioned that the divisions are synchronized up to a late stage in the echinoderms. This makes these eggs very convenient objects for the present kind of studies. The first echinoderm egg studied was that of the Swedish sea urchin, *Psammechinus miliaris*. In this egg each mitotic cycle is accompanied by an increase in respiration, the curve

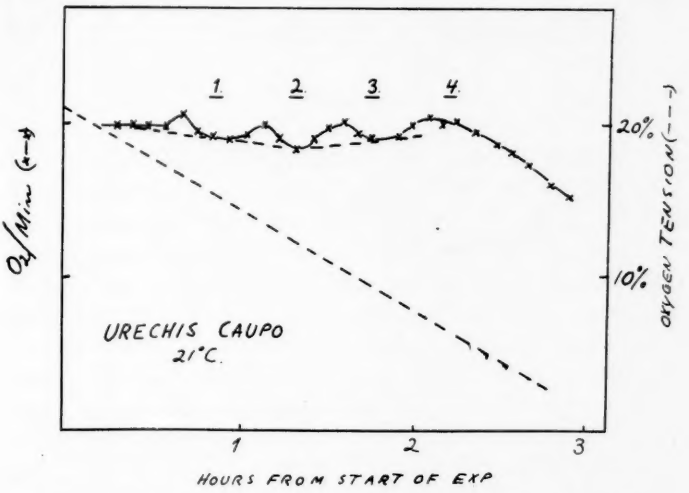

FIG. 3. *Urechis caupo*. Oxygen consumption shows a mitotic rhythm. Times of cytoplasmic division of the four first cell divisions indicated by numbers 1–4. Dotted line shows how the O_2-pressure (per cent. of an atm.) is decreasing during the experiment. At pressures lower than 6 per cent. (in the air bubble) the oxygen tension in the eggs is no longer sufficient to maintain normal respiratory rate. Ordinate in this and following figures are in arbitrary units.

for the early divisions looking very much the same as that for Urechis. However, it became apparent that the later division cycles (in which many more cells divide at the same time in the embryo than is the case in the earlier divisions) are accompanied by much larger respiratory variations. This is illustrated by the two curves (two different experiments) of Fig. 4. The reader may be confused by the fact that the general trend of this figure is to show a higher

respiratory rate in the first one to two hours of the experiment than might have been expected from the reports of other workers. It is a purely technical deficiency which manifests itself here (also appeared in exp. of Figs. 2 and 7). From the beginning of the experiment the water phase of the diver is always in equilibrium with the atmosphere, but in these experiments the gas in the diver is oxygen. The

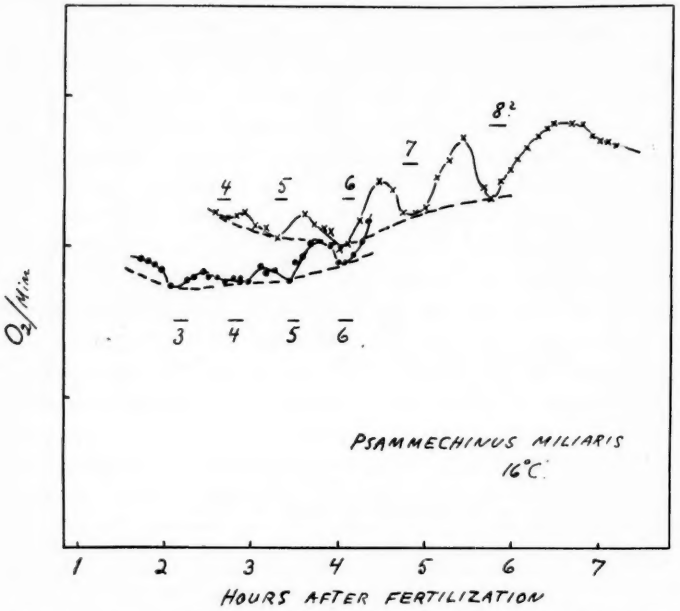

FIG. 4. Two experiments on the Swedish sea urchin, *Psammechinus miliaris*. Cleavage periods of cytoplasm indicated by numbers. Oxygen in divers.

water therefore takes up oxygen until a new equilibrium has become established within the diver. This results in an oxygen uptake which, of course, must be non-rhythmical and which is superimposed upon the rhythmical respiration of the eggs. In the first one to two hours of the present experiments this apparent respiration constitutes probably 10 to 20 per cent. of the total oxygen uptake measured, *i.e.*, it does not call for a serious revision of the first impression

one gets from Fig. 4 of the respiratory rhythm in the Psammechinus egg.

For the experiments on Urechis and Psammechinus a diver looking more or less like the one in Fig. 1, middle, was used. Because the gas phase is small (0.5–1.2 μl) in comparison to the number of eggs present in the diver, we get a very rapid change in equilibrium pressure of the diver (usually around 0.5–1.0–1.5 cm. H_2O/min.). For reasons to be mentioned later, it was desirable to increase the proportion air/gas in the diver by about ten times. This could only be done by increasing the air space of the diver, since the water phase could hardly be very much reduced. Let us consider the following case: The respiratory rate is kept constant. Then a certain increase in gas volume of the diver means a corresponding decrease in sensitivity (*i.e.*, the change of equilibrium pressure/min. gets smaller). But if at the same time we can measure the equilibrium pressure of the diver with a much higher accuracy than before, the sensitivity of the diver as a respirometer may remain unchanged despite the much increased air volume. Fortunately, the Cartesian diver is much more sensitive to pressure changes than thought before. Until recently we have only tried to measure equilibrium pressure to about $\pm$ 0.3 mm. H_2O; however, by using a very sensitive manometer of a special design, very small changes in equilibrium pressure can be picked up, the error being about $\pm$ 0.03 mm. H_2O. As soon as this became apparent the diver was remodelled from the one shown in Fig. 1, middle, into the one shown in Fig. 1, right. The latter diver, by the way, is much less fragile and therefore much more easy to handle than the former.

Fig. 5 shows an experiment on the eggs of the California sea urchin, *Strongylocentrotus franciscanus*. The experiment runs over 15 hours, all of which are hours of very busy and careful work. For this reason this egg does not lend itself to too much experimentation of this kind. The mitotic rhythm in O_2– uptake, however, seems to be well established. The egg of the Californian sand-dollar, *Dendraster excentricus* (another echinoderm), cleaves twice whenever

the Strongylocentrotus egg cleaves once. In the course of seven to eight hours from fertilization it hatches, and eggs in the diver hatch at exactly the same time as do control eggs in the same water-bath, only outside the diver. Fig. 6, upper curve (I), shows that the same respiratory rhythm as in the two other echinoderms can be demonstrated in the Dendraster egg.

I may several times in the above have called the demonstrated rhythm in oxygen uptake a "respiratory rhythm." Strictly speaking, this is not justified. The "respiratory" variations are not so big that it might not be possible to interpret the whole fluctuating curve in terms of a smoothly

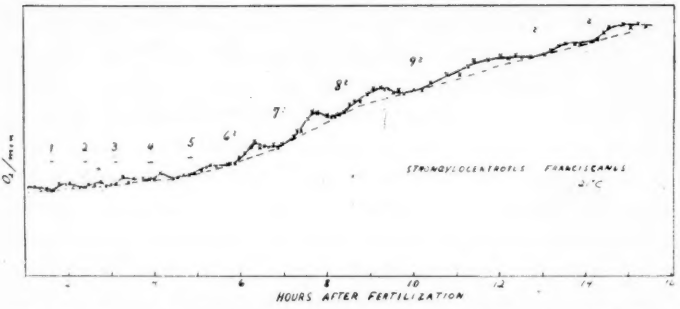

FIG. 5. *Strongylocentrotus franciscanus*. Mitotic rhythm in metabolism. Only the first five divisions could be actually observed in the diver. There are slight indications on the curve that the synchronicity of the divisions in the embryo is stopping after ninth division. Air in diver.

increasing respiration on top of which a reversible oxidation and reduction of some unknown chemical system is going on, the molecular O_2 being used in this process. This would simulate true variations in respiratory rate. The reason why I have been worrying about this is that Rapkine (1945) claims to have demonstrated a rhythmic (mitotic) variation in the detectable amount of SH– groups in the sea-urchin egg. Before we dare call the demonstrated mitotic fluctuations in oxygen uptake *respiratory* variations, it is required that it be demonstrated that the CO_2 output and the oxygen uptake fluctuate in the same rhythm. Here the reason why I wanted to increase the ratio gas/water in

the diver comes in: In the diver of Fig. 1, middle, a very significant part (65 per cent.) of the carbon dioxide will remain in solution in the water even if we do not absorb CO_2 in the neck seal and even if the water in the bottom droplet is slightly acid. In the diver shown in Fig. 1, right, the ratio gas/water is about 5, as compared to about 0.5 in the diver of Fig. 1, middle. It is easy to calculate that if the diver in Fig. 1, right, is tight to CO_2 (which it probably is) and at 20° C. (the temperature is not very significant)

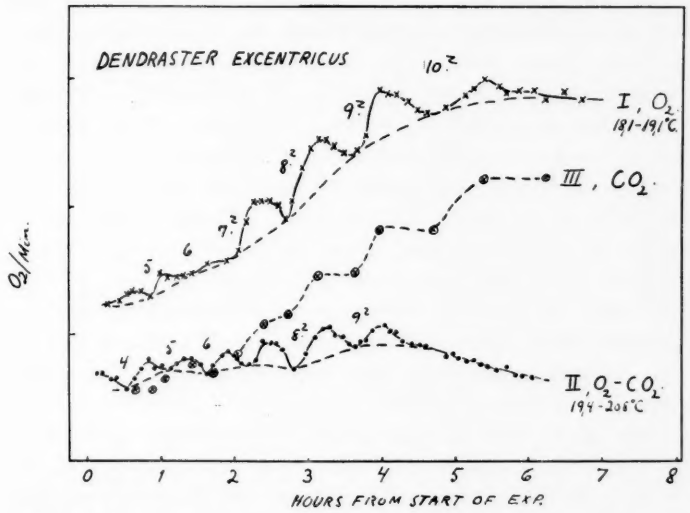

FIG. 6. *Dendraster excentricus.* Mitotic rhythm in oxygen uptake and in CO_2-output. Air in diver. Further explanation in text.

85 per cent. of the respiratory CO_2 will remain in the gas phase and therefore be manometrically measurable as the difference between the rates found with and without CO_2-absorption in comparable experiments.

Whereas the upper curve in Fig. 6 shows the variations in oxygen uptake in the Dendraster egg, the lower curve (II) shows the rate of pressure change when at the same time oxygen is consumed from and carbon dioxide is put out into the gas phase. By subtraction of the two curves we find the CO_2-production (curve III). This curve is interpreted

as indicating that the carbon dioxide production follows the same rhythm as does the oxygen uptake; in other words, *the rhythm found is a respiratory rhythm and therefore indicates a rhythmic energy output of the eggs.* Accordingly, these experiments give no evidence for assuming the presence of a reversible oxido-reduction process as proposed above. Admittedly the suggested rhythm in CO_2– output is not by any means as safe as is the statement that the oxygen uptake fluctuates in a mitotic rhythm. Three experiments have been run with carbon dioxide absorption in the diver, all giving identical results for the rhythm in O_2– uptake. Four experiments have been made in which the carbon dioxide was not absorbed. Two of these latter experiments were made in ordinary sea water, the other two were run in artificial bicarbonate free sea water. I could find no difference in the results in sea water and in artificial sea water. By subtracting each of the four experiments without CO_2– absorption from each of the three experiments with CO_2– absorption (12 subtractions) I invariably find that the resulting curve for CO_2– production shows a rhythm (as in Curve III) comparable to that demonstrated for the oxygen uptake. The carbon dioxide rhythm, however, can only be demonstrated for the large respiratory waves of the later division stages. A few words should be said also about the way the curves are subtracted, such as to give the CO_2– production: Seven different experiments, all on eggs from different females of Dendraster, are compared. This can only be done if we accept certain individual differences which exist on the curves. I have made the assumption, however, that curve maxima may safely be subtracted from curve maxima and curve minima from curve minima, provided, of course, that in each case mitoses having the same numbers are compared. This is what I did: I plotted the curves below one another and measured the *vertical* distance between corresponding maxima and minima. This vertical distance was plotted against time. The resultant curve of one such subtraction is the one shown in Fig. 6, III. No correction has been made for the amount of CO_2 (about 15 per cent. of the total CO_2) which

must have remained in solution in the water of the diver.

I certainly do not pretend definitely to have solved the CO_2– part of the problem discussed, but I hope to have presented a preliminary attack on a difficult problem, and I

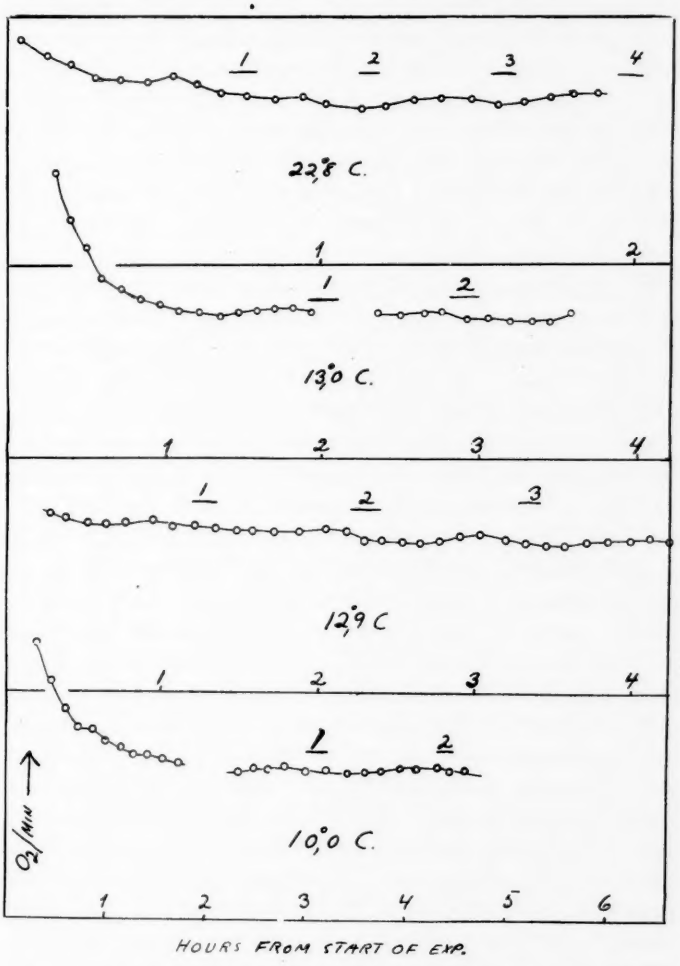

FIG. 7. *Urechis caupo*. Experiments at different temperatures. Slopes to the left in the figure due only to technique. Curves plotted in such a way that they can be directly compared. Wave phenomenon seems to be unaffected by temperature. All experiments with oxygen in the diver.

believe that the conclusion made is the best one to be drawn on the basis of the evidence available.

Next I would like to discuss a little more the shape of the respiration curve found in the five different eggs. I have not mentioned before that only for the first 4–5–6 divisions do we actually know that one mitotic cycle is represented by one respiratory wave. This is so because usually these first divisions—and only these—could be observed in the diver itself. With regard to later stages we are limited to mere guessing, but until we know better it is reasonable to assume that the larger respiratory waves found in all cases correspond to a mitotic cycle. If this is so, we must also assume (comp. the curves) that at the time when the echinoderms are hatching, the mitotic activity is slowed down extremely (by the way, hatching itself does not seem to be accompanied by any change in respiratory rate). As long as in an egg all blastomeres take part in each mitotic cycle, the number of blastomeres in the embryo must increase exponentially with the number of mitotic cycles. At the same time, the total volume of the embryo remains constant. Growth at the expense of outside material does not take place until much later. If we interpret our curves as indicating that each mitotic cycle is accompanied by an extra respiration (equal to the area between the sine curve and a smooth auxiliary curve which touches all valleys of the respiration curve), the question arises: Is the extra respiration a function of the total mass of the embryo or is it a function of the number of cells dividing per mitotic cycle. The answer is that most likely the extra respiration is the same for each early mitotic cycle, but only up to a certain stage (division No. 4–5 in Psammechinus, division No. 6–7 in Dendraster and in Strongylocentrotus, limit unknown for the frog and for Urechis because in these two eggs only the early divisions were studied). Up to this stage, therefore, the extra respiration is a function of the constant mass of the embryo rather than of the rapidly increasing number of cells dividing, and it is of the order of 2 per cent. of the total metabolism only. But after this stage has been passed, the extra respiration becomes more

closely a function of the number of cells dividing than of the mass of the embryo. In the first experiments with Psammechinus there was actually during these later stages a doubling of the extra respiration for each new mitotic cycle, the doubling beginning at the 5th division and ending after the 8th division. In other echinoderm eggs this proportionality between extra respiration and number of cell divisions taking place in the latter part of the segmentation period was not nearly so pronounced, but the principle of a larger and larger extra oxygen uptake for each new mitosis is the same (comp. the figures). The conclusion is drawn that a *physiological difference exists between the first 4–5–6 mitotic cycles and the later mitoses, at least in the echinoderm eggs considered, and perhaps more generally, too.* The later mitotic cycles are accompanied by extra respirations which may be, for instance, 10 per cent. of the total metabolism.

The experiments seem to indicate that each mitotic cycle in fertilized eggs is accompanied by an extra respiration, in other words, an extra energy output. In physiology we always like to find an extra work done whenever we find an extra energy output. With regard to the cell division we are utterly confused as soon as we try to define which work is being done. In one experiment on a fertilized frog egg, quite normal variations in oxygen uptake were found; probably the nuclei divided, but the cytoplasm did not. In another egg from the same batch which behaved the same way, the nuclei divided all right. Logically, we can not from this experiment deduce that the respiratory variations are not normally used for cleaving the cytoplasm. The only thing we can say is that the two processes are dissociable, and perhaps they have no connection at all. The fact that in the marine eggs studied the cytoplasm divides at a time when the respiration is already decreasing is a much better indication that the extra oxygen uptake is not used for "cleavage work," a concept which we must remember has never been physically defined.

One would much rather try to find some correlation between extra energy metabolism of the mitosis and some

kind of chemical work (spindle formation, other syntheses) which may well take place in the cytoplasm and at the same time be governed by the nuclei or at least timed in the same way as the mitotic cycle of the nuclei. So far, I have been unable, however, in the literature to find any chemical constituent of fertilized eggs, the growth of which could be said to follow a curve that can be correlated with the findings reported in this paper, or rather which can be correlated with the present findings as I have interpreted them. It may be important, however, that the overall metabolism of the eggs starts to rise more steeply at the time when each new mitotic cycle becomes associated with larger and larger extra oxygen uptakes. So the present status of this investigation seems to be that we have found evidence of an extra energy output during the mitosis, but so far we do not know what the cells do with this energy. To throw more light on this problem shall be the object for further studies.

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Discussion

Chairman Engle: Thank you very much. The paper is open to discussion.

Dr. MacGinitie: Isn't it possible that the manufacture of some chemical substance such as, for instance, chromatin, would correlate nicely with your results?

Dr. Zeuthen: I have considered, that since up to the stage of the sixth division (Dendraster) the extra oxygen consumption per mitotic cycle appears to be relatively constant, one must expect a substance which is formed by this extra respiration to grow with a constant rate up to this stage. After this, the extra respiration per mitotic cycle becomes bigger and bigger. So now the hypothetical substance should grow faster and faster. Little is known about the growth of chromatin in these early stages,[2] but something is known about the growth of the aggregate volume of the nuclei in the embryo (Conklin, 1935; Godlewski, 1935). Apparently this volume grows fast in the beginning, but after just about the sixth division (somewhat different in different eggs) the curve flattens out. After this, each nucleus tends to halve its volume at each division, so that the aggregate nuclear volume from now on constitutes an approximately constant fraction of the embryo. These facts do not seem to indicate a close relation between the formation of "chromatin" and the respiratory rhythm which we discuss. Admittedly, however, the aggregate nuclear volume may *quite possibly not be a good measure of anything important.*

Dr. Horowitz: In Urechis the first thing which happens after fertilization is that the polar bodies appear. Is this connected with any change in respiratory rate?

Dr. Zeuthen: As far as my experiments go there are two small peaks showing up on the curve at that time. The peaks have about the same height as those accompanying mitosis, but they are shorter in time.

[2] The recent paper by Villee *et al.* (*Jour. Cell. Comp. Physiol.*, June, 1949) shows that DNA in the Arbacia egg grows not too differently from this hypothetical substance. This changes my answer to Dr. MacGinitie. My results may perhaps correlate with the growth of "chromatin."

DR. TYLER: You didn't mention any experiments on unfertilized eggs, but I presume that they didn't show the peaks.

DR. ZEUTHEN: Experiments on unfertilized eggs of the frog and of Urechis have failed to show any peaks at all. In the frog, I find a quite gradual decrease in respiration, but in Urechis the respiratory rate of the unfertilized egg shows a gradual, very smooth increase.

DR. CHAMBERS: Is there any way of measuring the change in respiration immediately after fertilization with this method? Say, during the first 5, 10 or 15 minutes after fertilization?

DR. ZEUTHEN: Dr. Hans Borei (1948) in Sweden has recently published studies of this kind with the Cartesian diver. The principle is the same as that used for determining respiratory quotients (Needham *et al.*, Holter). By applying excess pressure on the diver for a very short time, a droplet containing eggs is forced down so as to come in contact with another droplet containing an activating agent. Some few minutes, however, may have to elapse before the diver " recovers " from this fairly rough treatment.

CHAIRMAN ENGLE: Are techniques available for a study of nucleic acid which could be compared with your curves?

DR. ZEUTHEN: No studies are available which permit a direct comparison of the thymo- or ribonucleic acid during early development with my curves. People have never bothered with sampling close enough for this.

DR. DELBRÜCK: It seems to me, as I understand it, that these extra oxygen uptakes are not correlated with the formation of new chromosome material. In that case, you would expect each successive bump to be twice larger than the preceding one, and evidence is strong that that is not so. Therefore, whatever the extra oxygen does, it is not directly correlated with the growth of nuclear components.

DR. ZEUTHEN: Only in one case (divisions 5–8 incl. in Dendraster) was each successive bump found to be twice the preceding one. The extra oxygen consumption accompanying earlier divisions (1–about 5–6) appears in all eggs to be independent of the number of cells dividing in the embryo.

The general picture does not seem to give a good correlation with the growth of nuclear components, so far as this growth is known to us.[3]

DR. TYLER: Concerning Dr. Delbruck's question: The total nuclear volume of the cleaving egg does not double at each division according to the figures of several investigators. It increases slightly.

DR. ZEUTHEN: The nuclear volume increases very slightly at that time of development, when I find approximately a doubling of the extra oxygen taken up per mitotic cycle. In earlier division stages when the extra oxygen is approximately constant from one mitotic cycle to the next, the total nuclear volume grows faster, but even during this period there may only in a very few cases be a doubling of the nuclear volume per cleavage. Conklin's curves differ somewhat from species to species.

DR. GIESE: I would like to hear how you compared those experiments on Urechis which were performed at widely different temperatures.

DR. ZEUTHEN: Of course, everything goes much faster at high temperature (22°–24° C.) than at low temperature (9°–10° C.); but if the curves obtained at different temperatures are plotted in such a way that they go as closely as possible on top of one another, I can see no certain difference between them. Differences may be present, of course, but in that case they are within the experimental error (comp. Fig. 7).

DR. TYLER: One curve showed a falling off of the rate of respiration when the oxygen tension dropped below 6 per cent. As I recall the data of Amberson, Tang and Gerard, there is no drop in the rate of oxygen uptake down to very low oxygen tensions.

DR. ZEUTHEN: In the experiment with Urechis eggs, the respiratory rate started dropping when the oxygen pressure in the air bubble went below 6 per cent. What is important in this connection is, of course, the oxygen pressure on those eggs which are most remote from the air bubble. This is

[3] Compare note, p. 319.

probably much lower and perhaps close to the figure Dr. Tyler remembers.

DR. DELBRÜCK: I heard you say, I think, that the time delay in diffusion is negligible. How big is it actually?

DR. ZEUTHEN: In the frog egg the mitoses follow one another with a period of around one hour, perhaps slightly less. For this egg Linderstrøm-Lang (1946) feels justified in fixing the limits for the possible delay to 0–7 minutes. The delay for O_2 and for CO_2 is not very different. Linderstrøm-Lang has not yet seen the results obtained for other eggs, but if I understand his mathematics rightly, the delay should in these experiments never exceed two minutes (period of respiration rhythm always larger than 25 minutes, comp. figures).

DR. PEASE: I wonder if this lag is so serious that for this reason you would not be able to identify, for instance, the rise in oxygen uptake with the breakdown of the nuclear membrane. Do you consider that the dissolution of the nuclear membrane initiates the rise in respiration?

DR. ZEUTHEN: I do not think that the delay is very important. For the evaluation of the experiments it is probably much worse that the scattering of the observed points makes it impossible to make a very accurate curve drawing. For this reason, I do not find it possible to answer the last point in your question affirmatively. The only thing I can say is that the first part of the mitosis is accompanied by a steady rise in metabolism. After metaphase-anaphase the respiratory rate again gradually decreases.

CHAIRMAN ENGLE: I have a feeling that this respiration is going to continue to rise and fall during the dinner and evening session. I think we will hold those stimulating data and bring this meeting to a close. I want to express from the standpoint of the National Research Council great appreciation for the care Dr. Tyler has taken in making arrangements for this meeting and for the participation of all you learned gentlemen, and especially to the Department of Biology at the California Institute of Technology, our gracious host.

Whereupon the meeting adjourned at 5:00 P.M.

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